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STUDIES ON THE PERCUTANEOUS ABSORPTION OF PARATHION AND PARAOXON

I. Hydrolysis and Metabolism Within the Skin

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During the last decade, parathion has been the most widely used organophosphorus insecticide. It has proved to be extremely valuable for crop protection and useful in combating communicable diseases. However, the wide spread use of this compound has also resulted in a great number of accidental intoxications, many of which have been lethal. The dermal route of absorption is usually thought to be important in such cases (for complete bibliography up to 1958 regarding percutaneous intoxications and relevant experimental studies, see Fredriksson, 1958). However, previous experimental studies on the percutaneous absorption of parathion have been limited almost exclusively to LD_{50}-determinations in various species. It was therefore decided to perform a series of investigations covering such subjects as metabolism within the skin, penetration into the skin, rate of percutaneous absorption, and decontamination of the skin surface. In this series paraoxon also has been included, partly because parathion is transformed into this compound *in vivo*, and partly because of the need for a reference substance.

Percutaneous absorption of organophosphorus cholinesterase inhibitors, such as DFP, sarin, parathion and paraoxon, involves special theoretical problems due to the fact that many of these compounds are readily hydrolyzed or in other ways metabolized in the tissues of the body. The immediate implication of this is that a percutaneously absorbed inhibitor might be inactivated or changed in other ways before it reaches the blood stream, if such metabolic processes occur within the cutaneous tissues. Thus, it has been shown by *in vitro* studies that guinea pig skin contains an enzyme, belonging to the group of phosphorylphosphatases, that is capable of hydrolyzing sarin and two closely related compounds; and studies in the cat *in vivo* also suggest that there is hydrolysis of sarin during its absorption through the cutaneous tissues, Fred-

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riksson, 1958. The last mentioned study indicated that a major part of the material was inactivated before it entered the blood stream.

When percutaneous absorption of parathion is considered, the possible hydrolysis within the skin must be taken into account, even though there is only indirect evidence of hydrolyzing enzymes in mammalian tissues, Casida, 1956. Parathion also might be subject to another metabolic process in the cutaneous tissues, namely its oxidation to paraoxon, an enzymic activation that occurs mainly in the liver, but also in several other organs, Kubištova, 1959. However, skin has never been tested for the presence of this enzyme. A transformation of parathion into paraoxon within the skin might have two different toxicological implications. If this more toxic metabolite is formed within the skin during percutaneous absorption of parathion, and if it undergoes no further change, this portal of entry might be particularly dangerous. However, paraoxon is readily hydrolyzed by phosphorylphosphatases, and the presence of such enzymes in the skin might instead decrease the toxicity of percutaneously absorbed parathion and, of course, paraoxon.

It was, therefore, decided to investigate in various species the possible occurrence of skin enzymes capable of metabolizing parathion and paraoxon, in order to obtain a proper foundation for evaluating future data regarding the percutaneous absorption of these two important compounds.

Methods

Tissue slices of liver were cut with the Stadie Riggs, 1944, tissue slicer; skin slices were cut as described previously, Fredriksson, 1958. The skin was trimmed carefully to remove excess subcutaneous tissues. The skin of experimental animals was removed immediately after sacrifice, that from humans was obtained from an abdominal area during surgery. None of the patients had a disease which would impair the structural integrity of the skin. The skin was kept in a moist chamber at 5° C for a few hours before testing or kept in the frozen state if longer storage was needed.

The hydrolysis of parathion (E 605, or diethyl 4-nitrophenyl thiononophosphate) or paraoxon (E 600, or diethyl 4-nitrophenyl phosphate) was measured by the Warburg method as described by Augustinsson and Heimburger, 1954 a, 1954 b, or by extraction and semi-quantitative estimation of the *p*-nitrophenol formed after separation on paper. The enzymic reaction was carried out at 25° C in 3 ml of total volume containing 0.0166 M and 0.12 M NaCl, 7.7×10^{-3}M paraoxon, or 7.2×10^{-3}M parathion, and 1 gm of skin slices or 0.1 ml of rabbit plasma in an atmosphere of nitrogen-carbon dioxide 95—5, V/V. The parathion or paraoxon was added with a micropipette to the side arm of the flask along with 0.6 ml of distilled water. After 10 minutes of equilibration at 25° C, the organophosphorus compound was dumped into the main compartment to start the reaction. A control flask that did not contain parathion or paraoxon was run also.

Two other control flasks each of which contained the organophosphorus compound, but not tissue, also were incubated. At the end of the reaction period, tissue was added to one of the flasks and both were acidified and extracted. These latter two flasks served to reveal any non-enzymic hydrolysis of the

organophosphorus compound during the incubation period or the extraction procedure.

At the end of the reaction period, 0.2 ml of 2.5 M hydrochloric acid was added to acidify the reaction mixture. The *p*-nitrophenol formed and the remaining organophosphorus compounds were extracted by shaking an aliquot of the reaction mixture 3 times with 4 volumes of ethyl ether each time. The ether extracts were evaporated at room temperature, and the residue was taken up in a 1.0 ml of ethanol. For the isolation of *p*-nitrophenol, the extract was chromatographed at 25° C on Whatman No. 1 paper in n-butanol saturated with 5 M ammonia as described by Erne, 1958. The *p*-nitrophenol, which moved about half way down the paper, readily separated from a large excess of parathion or paraoxon, both of which ran with the front. These compounds were revealed on the paper by spraying with 0.9 N KOH in ethanol and heating at 90° for 5 minutes as described by Karlog, 1957. The amount of *p*-nitrophenol formed was estimated semiquantitatively by comparing the spot size and color intensity with closely matching standards chromatographed at the same time as the extract.

The conversion of parathion to paraoxon in skin was carried out in a manner similar to that described by Davison, 1955, for liver. Skin or liver slices, weighing approximately 1 gm were shaken for 1 or 2 hours at 37° in an oxygenated atmosphere in 6 ml of reaction mixture containing Krebs-Ringer phosphate pH 7.4, 0.01 M glucose, 2.9×10^{-4}M parathion, and 3 percent ethanol.

The reaction mixture in the flask was acidified with 0.2 ml of 2.5 M hydrochloric acid and extracted three times with 1 volume of diethyl ether each time. The residues left after the evaporation of the ether were taken up in ethanol and chromatographed in the ethanol-water-chloroform solvent system on silicone impregnated Whatman No. 1 filter paper described by Metcalf and March, 1953.

The solvent system was modified from that of Metcalf and March by placing the immobile as well as the mobile phase in the chamber. This resulted in a better separation of *p*-nitrophenol and paraoxon. The movement of the paraoxon relative to *p*-nitrophenol is 0.88 in the unmodified solvent (Augustinsson and Jonsson, 1957) and 0.39 in the modified solvent.

Analytical grade parathion and paraoxon were obtained from the American Cyanamide Company. No *p*-nitrophenol could be detected in either compound, and the parathion was free of paraoxon when the respective compounds were chromatographed using a technique that permitted detection of less than 0.2 percent of the impurity.

A sample of parathion labelled with ^{32}P, which was used in other papers of this series, was also uncontaminated with paraoxon or *p*-nitrophenol. However, when autoradiograms were prepared from chromatograms developed in ethanol-chloroform-ammonia by apposition to Eastman Kodak No Screen Medical X-ray film, a faint spot that ran ahead of parathion and behind paraoxon was detected. This impurity could not represent more than 0.1 percent of the radioactivity in the parathion. The nature of the contaminant is unknown, but Augustinsson and Jonsson, 1957, have found that the *bis* (nitrophenyl ester) of ethyl phosphate has about the same R as the impurity in the unmodified solvent of Metcalf and March, 1953.

Results and Discussion

The results of the studies on the hydrolysis of parathion or paraoxon are presented in Table 1. Parathion was not hydrolyzed by the skin of any of the species tested. This organophosphorus compound is very sparingly soluble in water. It was, however, well suspended in the reaction mixture when the reaction vessel was shaken in the water bath in the Warburg apparatus. Attempts to increase the possible rate of hydrolysis of parathion by decreasing the size of the suspended particles of the phosphorus compound with the non-ionic detergents Sterox SE (a polyoxyethylene thioether) at 0.5 percent concentration or Triton X-100 (a monylphenoxy polyethylene oxide ethanol) at 1 percent concentration were unsuccessful even though stable suspensions of parathion were obtained under these conditions, and the hydrolysis of paraoxon by rabbit plasma was inhibited only 50 percent by these detergents. Also, the presence of the latter caused an apparent gas uptake by the skin. This spurious gas uptake may be related to the absorption of water by the skin slices with subsequent change in volume of the tissue. Because of these experimental difficulties, these detergents were not used in any of the other experiments reported in this communication.

As is shown in Table 1, paraoxon was hydrolyzed by slices of rabbit, cat, or human, but not rat skin. The rate was quite slow with human or cat skin. About 1 percent of the initial amount (6325 μg) of paraoxon was converted to *p*-nitrophenol in 90 minutes at 25° by 1 gm of the slices of skin of these two species. This low activity could be detected only by paper chromatography. On the other hand, the rabbit skin had much greater activity; about 20 percent of the initial amount (6325 μg) of paraoxon was hydrolyzed to *p*-nitrophenol under these conditions. With this species, it was possible to determine the activity of the skin slices on the same sample by both the chromatographic and Warburg manometric techniques. As can be seen in Table 1, there was fair agreement between the results obtained by the two methods.

The results of other studies carried out on slices of rabbit skin are presented in Table 2. An enzyme is probably responsible for the ability of this tissue to split paraoxon, since the activity is destroyed by heat and since it was a continuous reaction. Treatment of the rabbit skin before excision with a 0.1 percent solution of the antiseptic, zepharin, a benzalkonium chloride, under the same conditions used for preparation of the skin of patients for surgery, caused only about a 20 percent inhibition of this enzyme. Therefore, the low activity of the human skin that was obtained at operations probably was not a result of preoperative treatment of the skin. As is shown in Table 2, the activity of rabbit skin is readily leached out of the tissue by incubation in the reaction medium. Apparently, the enzyme is in the extracellular fluid or loosely bound to cellular structures. Another possibility is that the enzymic activity is due to the small amount of plasma that may be in the skin. There was, however, no difference in paraoxon splitting activity between bled and non-bled animals. Rabbit plasma does have much more paraoxon-splitting activity than do the other tissues of this animal. Liver was found to have 12 percent and skin only 0.7 percent of the enzymic activity of plasma. Aldridge, 1953, has reported similar relative activities of the liver and plasma of this species.

Table 1. *Hydrolysis of Parathion and Paraoxon by the Skin of Various Species.**

Species	Substrate	Warburg Manometric Method		Chromatographic Method		
		Comments	μM	Comments	μM	Molar Percent Conversion
Man	Paraoxon	No significant differences from skin without paraoxon were noted	—	After 225 min. of incubation; paraoxon blank 0.1 μM	0.7	3
	Parathion	No significant differences from skin without parathion were noted	—	After 225 min. of incubation the *p*-nitrophenol was at same concentration as parathion zero time	—	
Rat	Paraoxon	No significant differences from skin without paraoxon were noted	—	After 90 min. of incubation *p*-nitrophenol was at same concentration as paraoxon zero time	0	0
	Parathion	No significant differences from skin without parathion were noted	—	After 90 min. of incubation *p*-nitrophenol was at same concentration as parathion zero time	—	—
Rabbit	Paraoxon	Gas evolution increased with paraoxon	4.6	After 90 min. reaction time intense spot at position of *p*-nitrophenol	3.4	20
	Parathion	No significant differences from skin without parathion were noted	0	After 90 min. reaction time *p*-nitrophenol spot no darker than in parathion zero time	0	
Cat	Paraoxon	No significant differences from skin without paraoxon were noted	0	After 90 min. of incubation moderately intense spot at position of *p*-nitrophenol	0.2	1
	Parathion	No significant differences from skin without parathion were noted	0	After 90 min. of incubation no different from parathion zero time	0	

* In these experiments, finely minced skin of 1 gm wet weight was used. The dry weights were 29, 40, 36, and 33 percent of the wet weight of the human, rat, rabbit, and cat skin, respectively. The difference in dry weight is probably due to the small amounts of fur left on the skin of the rabbit, rat, or cat. The activity observed in the Warburg procedure was corrected for the non-enzymic hydrolysis of paraoxon and for the evolution of gas in the absence of paraoxon. The chromatographic method was corrected for the non-enzymic hydrolysis of paraoxon or parathion by means of the zero time controls described in the experimental section.

An attempt was made to differentiate between the enzymes present in the plasma and skin by determination of their respective Michaëlis-Menten constants for paraoxon according to the method of Lineweaver and Burk, 1934. It was found that the Michaëlis-Menten constants at 25° C for the paraoxon-splitting enzyme of plasma was 7.7×10^{-4}M and that for the enzyme present in the skin was 6.6×10^{-4}M. Because of the low activity of the skin and the variability of the carbon dioxide evolution from skin alone, the difference between the Michaëlis-Menten constants of these two tissues is probably within

Table 2. *Characteristics of the Factor in Rabbit Skin which Hydrolyzes Paraoxon.**

Experiment Number	Conditions	Activity μM CO_2 90 min.
1	Usual	3.2
	Heated at 80° for 10 minutes	0
2	Usual	3.70
	Skin treated with Zepharin antiseptic	2.90
3	Usual	4.15
	Skin incubated with reaction mixture 60 minutes and then removed before the paraoxon is added from the side arm of the flask	3.75

* In these experiments, 1 gm of rabbit skin was incubated for 90 minutes at 25° C in the Warburg apparatus as described in the experimental section. The observed activity is corrected for the non-enzymic hydrolysis of paraoxon and for evolution of gas from skin in the absence of paraoxon.

the experimental error of measurement of this constant in the skin. The results of these experiments are compatible with the view that the enzyme present in the rabbit plasma and in the skin are the same, but the results are not conclusive evidence since closely related enzymes may have the same Michaëlis-Menten constants. It was not possible to investigate this question with the skin of humans or cats because the activity was too low.

The objective of the next series of experiments was to determine whether the skin of various species contained enzyme systems for the conversion of parathion to paraoxon. Under the conditions used, the formation of paraoxon was readily demonstrated in liver slices as has been noted previously by Gersmann *et al.*, 1952; Gage, 1953; Metcalf and March, 1953; Aldridge, 1955; Davison, 1955; and Kubištova, 1959. With liver after 1 hour at 37° C, about 1 percent of the parathion was converted to paraoxon and another 2 percent was found as *p*-nitrophenol. The phenol probably arose by the hydrolysis of paraoxon, which was produced by the oxidation of parathion rather than by direct hydrolysis of the latter. Accordingly, about 3 percent of the parathion had been converted to paraoxon in 1 hour at 37° C. These findings are in agreement with the previous studies of Davison, 1955, and Kubištova, 1959. Under the same conditions, and even when the reaction period was extended to 2 hours, no evidence of the conversion of parathion to paraoxon by the skin of any of the species tested could be observed on chromatograms of tissue extracts under conditions in which a 0.2 percent conversion could have been detected. Skin slices have less ability than liver to promote the hydrolysis of paraoxon to *p*-nitrophenol. Therefore, paraoxon would have a greater chance to accumulate in the skin than in the liver if any enzyme that catalyzes the conversion of parathion to paraoxon were present. Under these conditions, the failure to detect paraoxon formation by the skin is even stronger evidence that the oxidation of parathion to paraoxon does not take place in this tissue.

The results suggest that parathion can be assumed to be absorbed in essentially

unchanged form. Thus, it could be expected that there will be no decrease in its toxicity during the very slow passage through the skin, and this lack of inactivation might make percutaneous absorption of parathion a particularly hazardous portal of entry. Paraoxon, on the other hand, can be hydrolyzed to quite an extent during its percutaneous absorption in three of the species tested. This hydrolysis should result in a relative decrease in the percutaneous toxicity of paraoxon in these species as compared with the rat. It is true that the enzymic activity in the skin is low compared with that of plasma, but percutaneous absorption is a very slow process while transportation of the toxic material within the blood stream is fast. Thus, inactivation within the skin might be of greater toxicologic importance. The results indicate that the skin of man and cat, which showed the lowest activity, was capable of splitting approximately 30 μg paraoxon per hour and gram of tissue at 25° C. This does not represent optimal activity, since the slices used were quite thick, and furthermore, the activity will, of course, be higher at body temperature. What seems to be a low activity might in reality be most important, as the rate of absorption of paraoxon has an order of magnitude of micrograms per hour and cm^2 (Fredriksson, 1961).

SUMMARY

The ability of the skin of man, cat, rabbit, and rat to hydrolyze or otherwise metabolize paraoxon (E 600, or diethyl 4-nitrophenyl phosphate) or parathion (E 605, or diethyl 4-nitrophenyl thiononophosphate) was investigated using the Warburg technique and paper chromatography. Parathion was not hydrolyzed or transformed into paraoxon by the skin of any of the species tested.

Paraoxon, on the other hand, was hydrolyzed by skin from man, cat, and rabbit. This reaction, which was enzymic in nature, occurred at the fastest rate in the rabbit. In this tissue, about 20 percent of the paraoxon was hydrolyzed in 1 hour by 1 gram of skin at 25° C, while only about 1 percent was converted by skin from man or cat.

The relation of these findings to the percutaneous absorption of these compounds was discussed.

RÉSUMÉ

La propriété de la peau de l'homme, du chat, du lapin et du rat d'hydrolyser ou de métaboliser d'une autre manière le paraoxon (E 600 ou diéthyl 4-nitrophényl phosphate) et le parathion (E 605 ou diéthyl 4-nitrophényl thiononophosphate), a été étudiée au moyen de la technique de Warburg et de la chromatographie sur papier. Le parathion n'a pas été hydrolysé ni transformé en paraoxon dans la peau d'aucune des espèces examinées. Par contre, le paraoxon est hydrolysé par la peau de l'homme, du chat et du lapin. Cette réaction, qui est d'origine enzymatique, s'effectue avec le plus de rapidité chez le lapin. Chez cet animal, le 20 % environ du paraoxon est hydrolysé en une heure par un gramme de peau à 25°, alors que la peau de l'homme et du chat n'en transforme que le 1 % seulement.

L'auteur a discuté la relation existant entre ces résultats et l'absorption percutanée de ces produits.

ZUSAMMENFASSUNG

Die Fähigkeit der Haut von Menschen, Katzen, Kaninchen und Ratten, Paraoxon (E 600, Diäthyl-4-nitrophenyl-phosphat) oder Parathion (E 605, Diäthyl-4-nitrophenyl-thiononophosphat) zu hydrolysieren oder auf andere Weise abzubauen, wurde in der Warburg-Apparatur und mit Hilfe der Papierchromatographie untersucht. Es zeigte sich, dass Parathion in der Haut der untersuchten Spezies nicht in Paraoxon umgewandelt wird.

Andererseits wurde Paraoxon von der Haut von Menschen, Katzen und Kaninchen hydrolisiert. Die Reaktion, die enzymatischer Natur war, verlief am schnellsten bei Kaninchen; ungefähr 20 % des Paraoxons wurden in 1 Stunde durch 1 g Haut bei 25° C hydrolisiert, während die Umsatzrate in der Haut von Menschen und Ratten unter den gleichen Bedingungen nur 1 % betrug.

Die Beziehungen dieser Befunde zur percutanen Resorption dieser Verbindungen wird diskutiert.

RESUMEN

Utilizando la técnica de Warburg y la cromatografía de papel se investigó la capacidad de la piel del hombre, gato, conejo y rata para hidrolizar o metabolizar en otra forma el paraoxon (E 600 o dietil 4-nitrofenil fosfato) o el paration (E 605, o dietil 4-nitrofenil tiononofosfato). Ninguna piel de las especies ensayadas hidrolizó el paration ni lo transformó en paraoxon.

El paraoxon, por otra parte, fué hidrolizado por la piel humana, la de gato y la de conejo. Esta reacción, que fué de naturaleza enzimática, tuvo lugar con la mayor rapidez en el conejo. En éste, aproximadamente el 20 por ciento del paraoxon fué hidrolizado en 1 hora por un gramo de piel a 25° C., mientras que sólo se convirtió un 1 por ciento con piel humana o de gato.

Se discutió la relación de estos datos con la absorción percutánea de estos compuestos.

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